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General and local anaesthetics perturb the fusion of phospholipid vesicles

Adrian C. Simmonds * and Michael J. Halsey

Division of Anaesthesia, Clinical Research Center, Watford Road, Harrow, HA1 3UJ (U.K.)

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The effects of general and local anaesthetics on Ca^{2+}-induced fusion of negatively charged lipid vesicles have been investigated. Vesicles composed of phosphatidylcholine and phosphatidic acid (2:1 molar ratio) were induced to fuse using 5 mM free Ca^{2+}. Fusion, assessed by an increase in size using gel filtration techniques and confirmed by electron microscopy, displayed a dependence on Ca^{2+} and Mg^{2+} concentration and on temperature. The inhalational anaesthetics halothane, methoxyflurane and diethyl ether enhanced fusion as did the uncharged local anaesthetic benzocaine. In contrast, the charged local anaesthetics lignocaine and bupivacaine inhibited the fusion process. It is suggested that the enhancement observed with the inhalational anaesthetics and benzocaine was mediated by an effect on lipid fluidity and the inhibition observed with the charged tertiary amine anaesthetics was due to an antagonism towards Ca^{2+}.

Introduction

The interaction between anaesthetics and lipids has been central to much of the work concerning anaesthetic mechanisms [1]. Early studies showing a correlation between anaesthetic potency and olive-oil solubility have recently been extended to show a correlation between potency and solubility in a lipid bilayer [2]. However, care must be taken to make a distinction between general and local anaesthetic mecanisms which involve synaptic transmission and axonal conduction, respectively. This is exemplified not only by the difference in concentration at which volatile anaesthetics produce these two effects [3] but also by the selectivity of anaesthetic alcohols of different length in producing either synaptic or axonal block [4].

One area which has received much attention is the effect of anaesthetics on lipid thermotropism. Despite differences in lipid composition and anaesthetic concentration, the overall picture which emerges from these studies is that anaesthetics tend to fluidise membranes and lower the gel to liquid-crystalline phase transition temperature of pure lipid species [5]. Exceptions to this generalisation include a dependency on cholesterol content [6] and measurements made in the presence of long-chain alcohols which themselves show a dependency on phospholipid structure [5]. With regard to the tertiary amine local anaesthetics, it has been determined that both the charged and uncharged anaesthetic species have nerve-blocking properties [7] but it is predominantly the uncharged forms which bind more strongly and have the major effect on the fluidization of the lipid bilayer [8]. However, these charged local anaesthetics have been shown to be antagonistic to calcium in anionic lipid membranes [9].

We have investigated the effects of volatile gen-

* Present address: C.R.C. & Wessex Regional Medical Oncology Unit, CF99, Southampton General Hospital, Southampton SO9 4XY, U.K.

Abbreviation: Mes, 4-morpholineethanesulphonic acid.

eral anaesthetics and charged and uncharged local anaesthetics on calcium-induced fusion of negatively charged lipid vesicles. In view of the controversy associated with the relevance of liposome fusion to biological membrane fusion [10], we have chosen a lipid composition which is subject to controlled fusion and is reported to reduce the leakage of internal aqueous contents to a minimum [11]. The methods we have used to demonstrate fusion have included size determination by gel filtration techniques and electron microscopy.

Materials and Methods

Materials. Egg yolk phosphatidylcholine and phosphatidic acid were obtained from Lipid Products in chloroform/methanol and the concentration determined by phosphate analysis. Sepharose 2B-CL was from Pharmacia and Mes from Sigma. All other reagents were AnalaR grade.

Lipid vesicle preparation. A solution of 35 μmol phosphatidic acid/70 μmol phosphatidylcholine in chloroform/methanol was dried down by rotary evaporation and then under vacuum at 0°C for at least 10 h. Liposomes were then formed by the addition of 2 ml buffer (2 mM Mes/10 mM NaCl/1 mM EDTA/0.02% NaN_3 (pH 6.1)) and shaking by hand at 37°C for 10 min. After leaving at room temperature for 1 h or at 4°C overnight, the suspension was then sonicated using a 19 mm diameter probe in an MSE 100 watt Ultrasonic Disintegrator at 20–23°C for eight periods of 2 min. Titanium particles were removed by centrifugation on an MSE Junior centrifuge at 3000 rpm for 15 min. All procedures were carried out under nitrogen.

Purification of the small unilamellar vesicles formed was achieved by elution through a Sepharose 2B-CL 30 × 1.5 cm column using deoxygenated buffer (2 mM Mes/10 mM KCl/1 mM EDTA/0.02% NaN_3 (pH 6.1)). Fractions containing small unilamellar liposomes were collected and pooled, equivalent to a total volume of approx. 7.5 ml. The lipid concentration in this pooled suspension varied from 8.6 to 9.6 mM by inorganic phosphate analysis.

Fusion experiments. Fusion experiments were carried out with 1 ml aliquots of the pooled vesicle suspension. Calcium was added to a final concentration of 6 mM. Since the buffer contained 1 mM EDTA, a calcium concentration of 5 mM was assumed to be available in the medium for interaction with phospholipid. Incubation of the lipid/calcium mixture was carried out at 37°C for 30 min with gentle vortexing every 5 min. At the end of this period, EDTA was added to a final concentration of 7.5 mM. The fused vesicles were then eluted through a Sepharose 2B-CL column as before but with buffer containing NaCl instead of KCl. The absorbance of the eluate was recorded continuously, allowing an estimate of the position of the void volume and of the elution volume of the treated vesicles. The use of eluate absorbance to monitor liposome elution was confirmed by phosphate analysis. An estimation of the total volume was made possible by collecting fractions and determining their potassium ion content by flame photometry. (Since the original 'purifying' elution had been carried out in potassium buffer and the subsequent analytical elution had been carried out with sodium buffer, a peak of potassium ion content within the fractions provided an estimate of the total volume.)

After determining the position of the three elution peaks in units of time, an index of size was calculated as follows:

$$\text{Size index} = \frac{V_t - V_e}{V_t - V_0}$$

where V_t = total volume; V_e = vesicle elution volume and V_0 = void volume. Fig. 1 shows a representative trace.

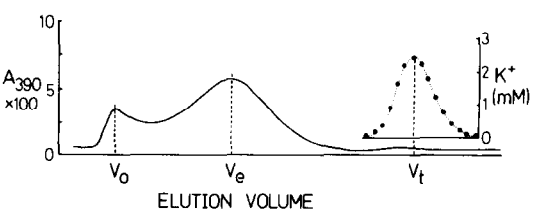

Fig. 1. Elution profile from a 50 ml Sepharose 2B column of fused lipid vesicles containing 9.0 μmol phospholipid consisting of phosphatidylcholine and phosphatidic acid in a 2:1 molar ratio. The void volume (V_0) and the vesicle elution volume (V_e) are given by the absorbance trace and the total volume (V_t) is given by a peak of potassium ion concentration.

$V_t - V_e$ provides an indication of the size and $V_t - V_0$ compensated for variations in elution conditions.

Additional evidence of fusion was obtained from electron microscopy using liposomes negatively stained with 2% ammonium molybdate.

Administration of anaesthetic. The inhalational anaesthetics halothane, methoxyflurane and diethyl ether were administered as a continuous flow of vapour in nitrogen delivered from a clinical vaporiser. Vesicle suspensions were equilibrated with the gas for 20 min before calcium addition and also during the incubation period with calcium. Samples of gas were analysed by gas chromatography to determine the actual concentration of anaesthetic. The local anaesthetics lignocaine, bupivacaine and benzocaine were added to the vesicle suspension in solution immediately before calcium addition.

Control experiments ensured that anaesthetics neither induced fusion without the aid of calcium nor affected the elution pattern from the Sepharose column.

In order to determine (a) the form of the relationship between inhalational anaesthetic concentration and liposome size index after treatment with calcium, and (b) the regression lines of best fit to this data, a computer analysis was performed using the programme GLIM.

Results

Fig. 2 shows the difference in elution pattern observed on prior incubation of liposomes with calcium. The difference in absorbance between the treated and untreated liposomes is due to differences in light scattering as a result of size differences, as is the slight discrepancy in peak positions from absorbance and phosphate analysis [12]. Pre-incubation with calcium causes the liposome elution peak to move towards the void volume, indicating an increase in size, quantified by the increase in size index from 0.403 ± 0.01 to 0.610 ± 0.034 (values $\pm$ S.D.). This was further demonstrated by electron microscopy. Figs. 3 and 4 show liposome preparations before and during incubation with calcium. Before fusion, the vesicles have a diameter of approx. 40–50 nm and are unilamellar. On incubation with calcium, two liposome

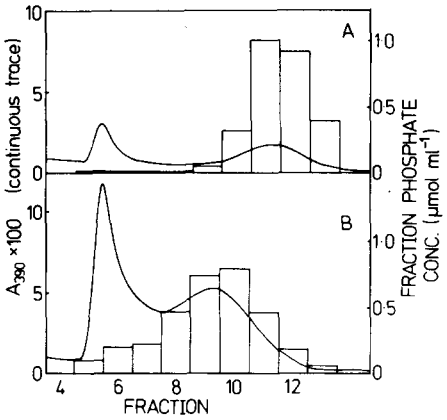

Fig. 2. Elution of lipid vesicles through a Sepharose 2B column and comparison of the lipid phosphate content of the column eluate fractions with a continuous absorbance trace. (A) Unfused liposomes; (B) Liposomes preincubated with 5 mM free Ca^{2+} for 30 min at 37°C.

populations are observed. The first of these consists of large structures which probably result from the fusion of a large number of vesicles. They are multilamellar but appear to be bounded by an uninterrupted bilayer. They are also characterised by narrow 'bridges' which may link two or more lobes of the structure and which are not seen in unfused liposome preparations. The second population is comprised of smaller liposomes, probably resulting from the fusion of a small number of

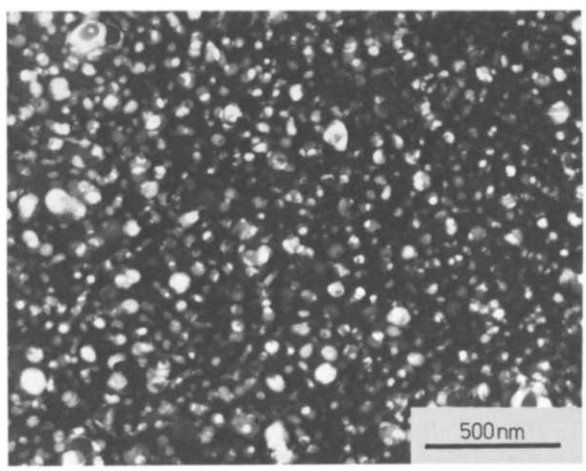

Fig. 3. Lipid vesicles negatively stained with 2% ammonium molybdate prior to fusion.

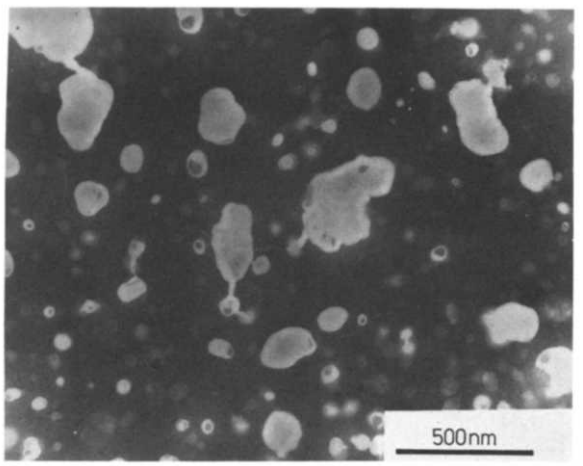

Fig. 4. Liposomes negatively stained with 2% ammonium molybdate after incubation with 5 mM free Ca^{2+} for 10 min.

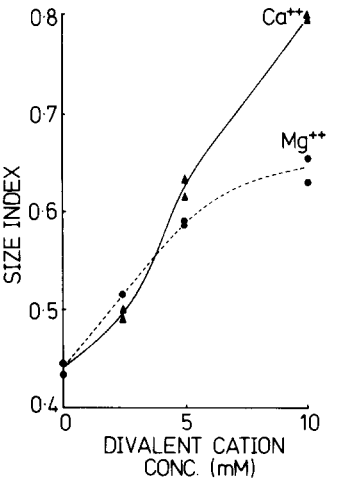

Fig. 5. Effects on vesicle fusion of varying incubation concentrations of Ca^{2+} and Mg^{2+}. Incubations were performed at 37°C for 30 min.

vesicles. The changes observed on incubation with calcium are not reversed on addition of EDTA. It is suggested that the large structures observed under electron microscopy are excluded from the gel filtration column and contribute to the absorbance peak in the void volume. The smaller liposomes which are more numerous constitute the absorbance and phospholipid peak included in the column. These liposomes have probably undergone fusion in a more controlled manner than the larger structures. The diameter which might be expected to accrue on the fusion of two vesicles, each having a diameter of 50 nm would be 70 nm (assuming a spherical product). In view of the limitations of this technique, this difference would not readily be seen.

The calcium and magnesium concentration dependence was determined using the concentrations as shown in Fig. 5, in addition to the 1 mM assumed to interact with the 1 mM EDTA present in the medium. EDTA was added after 30 min to a concentration 1.5-times that of the divalent cation. The highest concentration corresponds to a divalent cation to total lipid ratio of 1.1 : 1 or a divalent cation to phosphatidic acid ratio of 3.3 : 1.

The temperature dependence of fusion between 20 and 37°C is shown in Fig. 6. The conditions of the above experiments were otherwise as described earlier.

In the anaesthetic experiments, changes in size index were calculated using control vesicles from the same vesicle preparation. Differences in size index with and without anaesthetic in the absence of calcium were 0.0032 ± 0.0036 (mean ± S.D.).

The effects of the inhalational anaesthetics halothane, methoxyflurane and diethyl ether in the presence of calcium are shown in Fig. 7. The curves are produced by the curve-fitting procedure

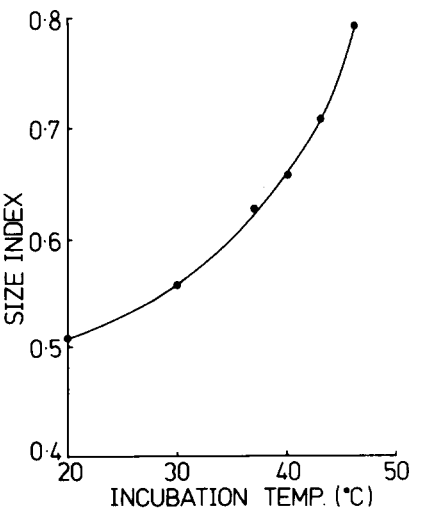

Fig. 6. Effect of temperature of incubation with Ca^{2+} on vesicle fusion. Incubations were performed for 30 min in the presence of 5 mM free Ca^{2+}.

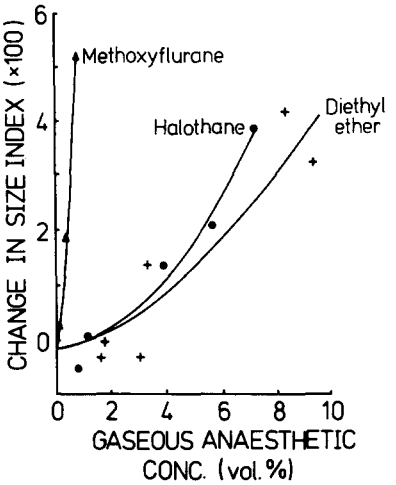

Fig. 7. Effects of halothane (●), methoxyflurane (▲) and diethyl ether (+) on vesicle fusion, induced by incubation with 5 mM free Ca^{2+} for 30 min at 37°C.

incorporated in the computer programme GLIM. They are significantly different by analysis of variance at the 0.1% level. Experiments using high concentrations of anaesthetic (20–25%) brought about size indices of 1.0 and were not included in the analysis. Enhancement of fusion was observed

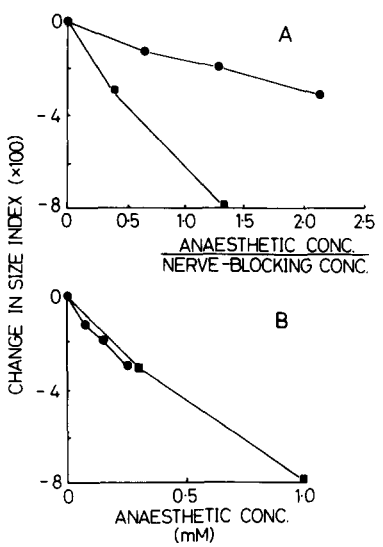

Fig. 8. Comparison of the effects of the local anaesthetics lignocaine (■) and bupivacaine (●) on vesicle fusion. (A) Anaesthetic concentration expressed relative to the nerve-blocking concentration at pH 6.1 [24]. (B) Anaesthetic concentration expressed as added aqueous concentration.

TABLE 1

TIME DEPENDENCY OF CHANGE IN SIZE INDEX WITH BENZOCAINE

Liposomes were incubated with 5 mM free calcium in the presence of 2.0 mM benzocaine.

Time (min)	Size index
0	0.594
10	0.648
30	0.698

with all three inhalational anaesthetics, their efficiency to do this rising with their increasing potency in producing anaesthesia in man. Concentrations producing surgical anaesthesia in man are 0.16% for methoxyflurane, 0.75% for halothane and 1.92% for diethyl ether [13] all at atmosphere absolute.

In contrast to the inhalational anaesthetics, the tertiary amine local anaesthetics lignocaine and bupivacaine inhibited the fusion process. This was not related to nerve-blocking potency but rather to the aqueous concentration as shown in Fig. 8. At the pH used (pH 6.1), these molecules are predominantly charged (lignocaine $pK_a = 7.9$, bupivacaine $pK_a = 8.1$) [14]. In order to test the hypothesis that this result was related to the charge, a non-charged local anaesthetic benzocaine was tested and found to enhance fusion as did the inhalational anaesthetics (see Table I).

Discussion

The method we have used to demonstrate changes in liposome size is based on a gel filtration technique described by Liao and Prestegard [11].

The size index is related to a column partition coefficient (K_d) according to the equation:

$$\text{Size index} = 1 - K_d$$

A relationship between particle radius and a function of K_d has been postulated as follows [15]:

$$a = a_0 + b_0 erfc^{-1} K_d$$

where a = particle radius; $erfc^{-1}K_d$ = the inverse error function complement of K_d; a_0 and b_0 are constants for a particular gel.

However, using calibrating proteins of known Stokes' radius we were unable to verify this. Other workers also have found a non-linear relationship between particle radius and erf^{-1} $(1 - K_d)$ [16]. For these reasons, while recognising the limitations, we adhered to the empirical size index parameter as expressed in Materials and Methods.

Despite differences in presentation of data, our results are compatible with Liao's and Prestegard's data as far as the calcium/magnesium dependence [17] and the time dependence of fusion [11]. The electron micrographs give no indication that increases in size could have been due to aggregation (rather than fusion) of liposomes. The liposomes appeared to be bounded by an uninterrupted bilayer and the changes were not reversed on addition of EDTA.

The effects of anaesthetics on liposome fusion stress the importance of (a) lipid fluidity and (b) phospholipid-divalent cation interactions in membrane fusion. The use of liposome fusion as a model for the molecular events underlying membrane fusion is particularly useful because it is possible to manipulate the components of the system without affecting other processes which might be involved in biological membrane fusion.

Anaesthetics which increase fluidity were shown to enhance fusion. The effect of temperature on liposome fusion is consistent with this observation. The gel to liquid-crystalline phase transitions for the individual lipids are -15 to -7°C for egg yolk phosphatidylcholine [18] and 15 to 20°C for phosphatidic acid [19]. In the temperature range investigated, between 20 and 46°C, both lipids were above their phase transition temperatures. Other workers have shown that the addition of cholesterol to phosphatidylglycerol liposomes in the liquid-crystalline phase reduces their ability to fuse [20]. It is also interesting to note that fluidity changes have been detected in the membranes of naturally fusing cells [21].

The calcium/magnesium dependence of fusion showing a higher incidence of fusion at the higher concentrations with calcium than magnesium is interesting in relation to the known physiological dependence of membrane fusion processes on calcium [22]. The charged tertiary amine anaesthetics which are known to be Ca^{2+} antagonists [9,23] caused a reduction in the extent of liposome fusion. To confirm that this result was related to the presence of the positive charge on these anaesthetics, an uncharged local anaesthetic was investigated and shown to have the opposite effect as explained above. The charged local anaesthetics are well-suited to disrupt Ca^{2+} complexes with negatively charged phospholipid because as a result of their amphipathic nature, they lie in the lipid bilayer in such a way as to reduce the net negative charge on the surface of the bilayer [23]. The effects of the charged local anaesthetics were not related to their potency but purely to their aqueous concentrations. Dibucaine has been reported to have a similar inhibitory effect on Ca^{2+}-induced fusion of phosphatidylglycerol liposomes [19].

The relevance of the model of liposome fusion to the molecular events underlining general anaesthesia is not apparent. We have demonstrated that effects observed on liposome fusion are only significant at 3–5-times the concentrations required for general anaesthesia. It is possible that the lipid components of the present model do not mimick the site of anaesthetic action as well as other lipids might. Alternatively, it may be that anaesthetics act in vivo via protein interactions.

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